

Increased Expression of Keratin 16 Causes Anomalies in Cytoarchitecture and Keratinization in Transgenic Mouse Skin

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Abstract. Injury to epidermis and other stratified epithelia triggers profound but transient changes in the pattern of keratin expression. In postmitotic cells located at the wound edge, a strong induction of K6, K16, and K17 synthesis occurs at the expense of the keratins produced under the normal situation. The functional significance of these alterations in keratin expression is not known. Here, we report that overexpression of a wild-type human K16 gene in a tissue-specific fashion in transgenic mice causes aberrant keratinization of the hair follicle outer root sheath and proximal epidermis, and it leads to hyperproliferation and increased thickness of the living layers (acanthosis), as well as cornified layers (hyperkeratosis). The pathogenesis of lesions in transgenic mouse skin be-

gins with a reorganization of keratin filaments in postmitotic keratinocytes, and it progresses in a transgene level-dependent fashion to include disruption of keratinocyte cytoarchitecture and structural alterations in desmosomes at the cell surface. No evidence of cell lysis could be found at the ultrastructural level. These results demonstrate that the disruption of the normal keratin profile caused by increased K16 expression interferes with the program of terminal differentiation in outer root sheath and epidermis. They further suggest that when present at sufficiently high intracellular levels, K16, along with K6 and K17, appear capable of inducing a reorganization of keratin filaments in the cytoplasm of skin epithelial cells.

KERATINS are epithelial-specific intermediate filament (IF)¹ proteins encoded by a large multigene family. The ~30 proteins (mol mass 40–70 kD) can be subdivided into type I or acidic and type II or basic based on DNA sequence homology (Fuchs et al., 1981; Moll et al., 1982a). Since keratin filament assembly begins with the formation of a type I–type II heterodimer (Coulombe and Fuchs, 1990; Hatzfeld and Weber, 1990; Steinert, 1990), all epithelial cells express at least one member of each keratin sequence subtype. Keratin gene expression is tightly regulated in an epithelial tissue-type and differentiation-specific manner, although the underlying molecular basis is not well understood. The complex pattern of keratin gene expression occurring in stratified epithelia is well conserved among mammalian species (Moll et al., 1982a; Sun et al., 1984). Mitotically active basal cells express the type II keratin K5 and the type I keratin K14 (Nelson and Sun, 1983). Upon commitment to terminal differentiation, basal cells down-

regulate the synthesis of K5 and K14 (Fuchs, 1990) and up-regulate that of other combinations of type I and type II keratins. These combinations appear to be specific to the program of terminal differentiation being executed, and they vary between epithelial tissue types (Sun et al., 1984). In the epidermis of skin, for instance, the major differentiation-specific keratins are the type II K1 and the type I K10 (Fuchs and Green, 1980). Recent transgenic mouse studies have provided good evidence that the filaments built from the K5-K14 and K1-K10 pairs function to impart mechanical strength and establish cytoarchitecture in epidermal cells (Vassar et al., 1991; Coulombe et al., 1991a; Fuchs et al., 1992). In support of this, point mutations in either the K5 or K14 gene cause epidermolysis bullosa simplex (EBS) (Coulombe et al., 1991b; Bonifas et al., 1991; Lane et al., 1992), while mutations in the K1 or K10 gene cause epidermolytic hyperkeratosis (EHK) (Cheng et al., 1992; Chipev et al., 1992; Rothnagel et al., 1992). EBS and EHK are distinct skin blistering disorders with autosomal dominant inheritance, in which the basal and suprabasal layers of the epidermis, respectively, rupture in response to trivial mechanical trauma (See Fuchs and Coulombe, 1992). Additional evidence obtained in transgenic mice (e.g., Baribault et al., 1993) or other contexts (Newport et al., 1990; Janmey et al., 1991; Torpey et al., 1992) also point to a role for IFs in providing mechanical strength to cells and tissues.

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1. *Abbreviations used in this paper:* EBS, epidermolysis bullosa simplex; EHK, epidermolytic hyperkeratosis; IF, intermediate filament; ORS, outer root sheath.

Injury to stratified epithelial tissues, including epidermis, esophagus, cornea, and trachea, triggers profound changes in the pattern of keratin gene expression (e.g., Weiss et al., 1984). After wounding, or in disease states associated with tissue hyperproliferation, these epithelial tissues adopt a similar pattern of keratin gene expression (Moll et al., 1983; Sun et al., 1984). While the expression of K5 and K14 continues in the mitotically active cells of the tissue, a strong induction of the expression of keratins K6 (type II), K16, and K17 (type I) occurs postmitotically (e.g., Stoler et al., 1988; Kopan and Fuchs, 1989a) at the expense of the differentiation-specific keratins synthesized in the normal situation. After physical injury to human (Tyner and Fuchs, 1986; Mansbridge and Knapp, 1987; de Mare et al., 1990) and mouse epidermis (our own unpublished data), this "switch" in keratin gene expression occurs very rapidly, such that keratins K6 and K16 can be detected within 4–8 h at the edges of the wound. This makes this induction event one of the earliest known markers of the onset of tissue regeneration: for instance, the increase in mitotic activity that must take place at the wound edge does not begin before much later, i.e., at 20–24 h after trauma (Bereiter-Hahn, 1986; de Mare et al., 1990; our unpublished mouse data). Functional epidermal regeneration is known to occur in human EBS patients (Fine et al., 1991) and in transgenic mice expressing dominant mutants of K14 (Coulombe et al., 1991a), suggesting that intact K5-K14 filaments may not be required in this process (see Fuchs and Coulombe, 1992). Taken together, these elements suggest that keratins K6, K16, and/or K17 may play a unique role allowing stratified epithelial tissues to achieve functional regeneration. This possibility is made all the more intriguing by the recent demonstration that depletion of maternal cytokeratins from early xenopus embryos results in a loss of the compacted epithelial surface of the blastula, an inability to close a wounded surface, and a defective gastrulation (Torpey et al., 1992; see Klymkowsky et al., 1992).

We have initiated studies aimed at understanding the functional significance of the remarkable changes in keratin gene expression occurring after tissue injury and in several diseases states. Here, we report that the overexpression of a wild-type human K16 gene in a tissue-specific fashion in transgenic mice results in aberrant keratinization of the hair follicle outer root sheath and adjacent epidermis, with hyperproliferation and increases in the thickness of the living layers (acanthosis) and cornified layers (hyperkeratosis). These studies support the notion that the architecture of the keratin filament network is a determinant of keratinocyte cytoarchitecture, and that perturbation of the latter (in the absence of cytolytic damage) is sufficient to significantly alter the program of terminal differentiation in stratified squamous epithelia.

Materials and Methods

Production and Screening of Transgenic Mice

A previously characterized human genomic clone containing the entire K16 gene (Rosenberg et al., 1988) was used for transgenic mouse studies. Upon HindIII digestion, an 11-kb DNA fragment comprising ~6 kilobase of 5' upstream sequence, the K16 gene including all the introns, and ~1,500 bp of 3' noncoding sequence was generated and prepared for microinjection into fertilized mouse embryos. After overnight incubation in Whetten's medium, viable, two cell-stage microinjected eggs were reimplanted into the oviduct of pseudopregnant mothers and carried to term. 3 wk after birth,

pups were weaned, anesthetized, and the distal 1 cm of the tail severed. Genomic DNA was extracted, restricted with BamHI, and subjected to agarose gel electrophoresis and Southern analysis according to standard procedures. To detect the human K16 transgene, we used a 3.1-kb fragment derived from within the 5' upstream sequence of the transgene construct that does not hybridize to control mouse DNA. All mice used were from Jackson Labs (Bar Harbor, ME): C57B6/BalbC3 (agouti) mice were used for the production of transgenic animals and their breeding, and in addition, FVB/NJ (albino) mice were also used for breeding.

Production and Characterization of an Antiserum Directed against Human Keratin 16

A 21-mer peptide, NH₂-C-Q-T-R-P-I-L-K-E-Q-S-S-S-S-F-S-Q-G-Q-S-S-COOH, corresponding to the K16 carboxy-terminal sequence predicted from the human gene (Rosenberg et al., 1988), was synthesized, purified, and conjugated to maleimide-activated keyhole limpet hemocyanin carrier as described by the manufacturer (Pierce Chemical Co., Rockford, IL). A polyclonal antiserum was produced in rabbits according to established procedures (Hazelton Research Products Inc., Denver, CO). Bleeds were tested by immunoblotting using purified human recombinant keratins, as well as keratin extracts prepared from human epidermal cells. Our analyses established that the rabbit antiserum used in this study is monospecific for K16 among human epidermal keratins (data not shown).

Intermediate Filament Protein Extraction and Analysis by Electrophoresis and Immunoblotting

Intermediate filament proteins were isolated as described (Wu et al., 1982). The final Triton X-insoluble pellet was solubilized in a 50 mM Tris-HCl buffer containing 8 M urea and 5% β -mercaptoethanol. Protein concentration was determined by the Bradford assay (Bradford, 1976) using reagents purchased from Bio-Rad Laboratories (Richmond, CA). Intermediate filament proteins were resolved using SDS-PAGE (8.5% acrylamide) and either stained with Coomassie blue or electroblotted onto nitrocellulose as described before (Coulombe et al., 1991a). For immunoblotting, antisera used included the rabbit polyclonal anti-human K16 produced as described above, a rabbit polyclonal anti-mouse K6 (Choi and Fuchs, 1990), a rabbit polyclonal anti-K14 (Stoler et al., 1988), AE1, a mouse monoclonal antibody (Boehringer Mannheim Biochemicals, Indianapolis, IN), which recognize epidermal type I keratins (Woodcock-Mitchell et al., 1982), and K8.12, a mouse monoclonal anti-K16, -K13, and -K15 antibody (Sigma Immunochemicals, St. Louis, MO). Bound primary antibodies were detected with alkaline phosphatase-coupled goat anti-rabbit or goat anti-mouse IgGs (Bio-Rad Laboratories).

Morphological Studies

For routine light microscopy studies, tissues were fixed in Bouin's and embedded in paraffin. 5- μ m sections were either stained with hematoxylin and eosin, or were processed for immunohistochemistry using the silver-enhanced immunogold procedure as recommended by the manufacturer (Amersham Corp., Arlington Heights, IL). In addition to those already mentioned above, primary antisera used included a rabbit polyclonal anti-filaggrin (Dale et al., 1985), a rabbit polyclonal anti-desmoplakin I (Stappenbeck and Green, 1992), and K8.60, a mouse monoclonal anti-K10 and K11 (Sigma Immunochemicals). Sections were also prepared from fresh skin frozen in dry ice-cooled isopentane, and labeled by indirect immunofluorescence according to standard procedures. Bound primary antibodies were detected with an FITC-conjugated goat anti-rabbit IgG secondary (Vector Labs, Inc., Burlingame, CA), or a biotin-conjugated goat anti-mouse IgG secondary (Kirkegaard & Perry Laboratories, Gaithersburg, MD) followed by a streptavidin-Texas red conjugate (Vector Labs, Inc.). Finally, to identify mitotically active cells, mice were injected intraperitoneally with bromodeoxyuridine (BrdU) 2 h before death. Tissues were paraffin embedded, and 5- μ m sections were immunostained with a mouse monoclonal anti-BrdU antibody (Caltag Labs, San Francisco, CA).

For routine electron microscopy studies, tissues were fixed in buffered 4% paraformaldehyde and 1% glutaraldehyde, post-fixed in 1% osmium tetroxide, and embedded in LX112 epoxy-resin (Ladd Research Industries, Inc., Burlington, VT). For immunoelectron microscopy, tissues were fixed in buffered 3% paraformaldehyde and embedded in Lowicryl K4M at -20°C as previously described (Bendayan, 1989). Sections were immunostained with primary rabbit polyclonal antisera directed against K16 and

K14 (described above), followed by 10 nm gold-conjugated goat anti-rabbit antibody (Amersham Corp.). The procedures used and the specificity controls performed were as described (Coulombe et al., 1989).

Results

Transgenic Mice Harboring Several Copies of the Human K16 Gene Develop Severe Lesions in Skin

To investigate the consequence of raising the intracellular concentration of K16 in skin and other epithelial tissues, we introduced the intact human K16 gene into the germline of mice. The DNA construct used, a cloned 11-kb human genomic DNA insert containing the entire K16 gene (Rosenberg et al., 1988), is represented in Fig. 1 A. The coding segment within this genomic code corresponds to that of the human K16 cDNA obtained by RT-PCR from total mRNA prepared from human epidermal keratinocytes in primary culture (Paladini, R., and P. A. Coulombe, unpublished data). Transient expression of the 11-kb genomic construct (subcloned into a cytomegalovirus promoter-based vector) in PtK2 epithelial cells resulted in the incorporation of the transgene product into the preexisting keratin filament network (data not shown), confirming the original report by Rosenberg et al. (1988). Five founder transgenic mice having integrated between <1 and ~10 copies of the human K16 gene in their genome were produced and further analyzed.

Two transgenic founders (mouse 4-12 and 5-7, both ~8-10 transgene copies; see Fig. 1 B) developed skin lesions starting at 6-8 d after birth, coinciding with the appearance of hair. These lesions were initially manifested as thickening

and scaling of the skin on an erythematous base, and they preferentially affected the tail, ear, neck, pelvic region, and other axillary areas. In founder mouse 4-12, a female, the lesions thereafter progressively became more severe within 2-3 wk, with crusted erosions covering a significant portion of the ventral surface. Mouse 4-12 died at 2 mo of age without successful mating. In founder mouse 5-7, a male, the initial lesions progressively healed and had disappeared after 1 mo. The same pattern of skin lesions appearing at 6-8 d after birth followed by slow healing was seen in heterozygous transgenic offsprings produced from founder 5-7. An exception to this is tail skin, for which the lesions tended to be more severe, and left permanent mutilating scars in a subset (~25%) of heterozygous transgenic offsprings. A small number of adult transgenic mice were characterized by a recurrence of the skin lesions affecting the neck and/or pelvic region. On the other hand, transgenic founders with lower transgene copy number, such as 4-14 (~3 copies), 6-17 (~2 copies), and 5-13 (mosaic mouse with <1 copy), as well as their F1 heterozygous offsprings (Fig. 1 B), have not featured skin lesions since birth. The 4-14 and 6-17 lines were bred to homozygosity (~4-6 transgene copies; Fig. 1 B), but failed to show the skin phenotype. As shown below, these nonphenotypic transgenic animals have low levels of transgene product in the relevant epithelial tissues.

To establish that the skin phenotype was a direct consequence of transgene expression, we examined the effects of increasing further the transgene copy number in the mildly phenotypic 5-7 line through mating the line to homozygosity (~16-20 copies; Fig. 1 B). These animals rapidly developed skin lesions within a few days after birth (Fig. 1 C), and in

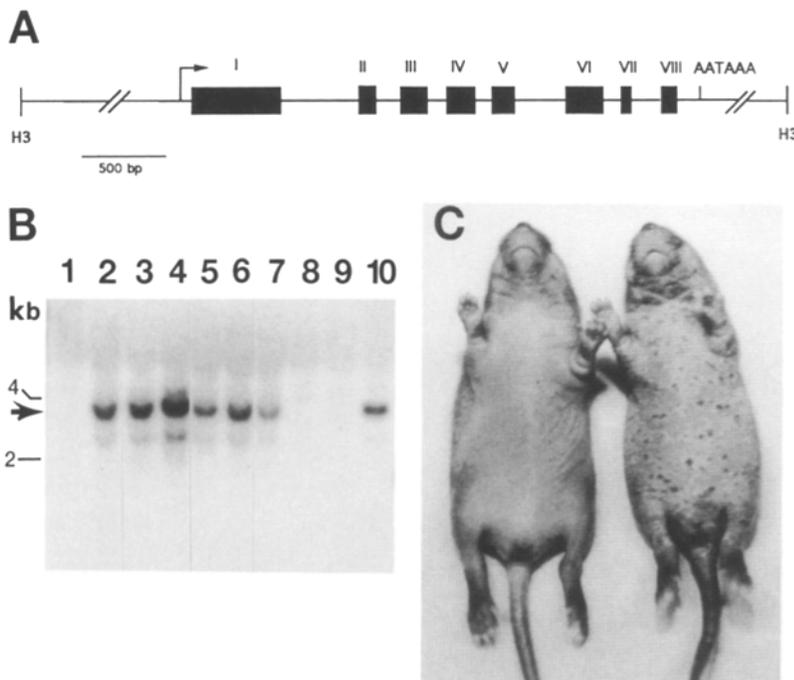


Figure 1. Production and identification of transgenic mice overexpressing the human K16 gene. (A) Schematic of the DNA construct. The DNA microinjected in mouse embryos consisted in a cloned 11-kb human genomic DNA fragment (Rosenberg et al., 1988) comprising ~6 kb of 5' upstream sequence, the K16 gene including all eight exons (roman numerals) and seven introns (~3.5 kb), and ~1500 bp of 3' noncoding sequence. The arrow indicates the position of the transcriptional start site, while AATAAA depicts the position of the polyadenylation signal. (B) Southern blot analysis of mouse tail DNAs. ~12 μ g of genomic DNA was digested with Bam HI, and subjected to agarose gel electrophoresis and Southern analysis according to standard procedures. To detect the human K16 transgene, we used a 3.1-kb fragment derived from within the 5' upstream sequence of the 11-kb DNA construct. Lane 1, Nontransgenic mouse control; lane 2, founder 4-12; lane 3, F1 heterozygous offspring, line 5-7; lane 4, F2 homozygous offspring, line 5-7; lane 5, F1 heterozygous offspring, line 4-14; lane 6, F2 homozygous offspring, line 4-14; lane 7, founder 6-17; lane 8, founder 5-13; lane 9, nontransgenic mouse control; lane 10, nontransgenic mouse control to which 50 pg transgene was

added. Note that founder 5-13 is a mosaic, and that a positive signal is seen upon longer exposure of the blot. (C) Distribution of skin lesions on the ventral surface of phenotypic transgenic mouse. The animal on the right is an 11-d-old 5-7-F2 homozygous mouse (see lane 4 in B), while the left one is a nontransgenic littermate. Notice the presence of darker papules of ventral skin, and of thickened skin with a crusty appearance on the neck and in the pelvic area. Tail skin is also severely affected.

most cases died within 2 wk. In addition, doubly heterozygous transgenic mice were produced through matings involving F1 heterozygous animals from the 5-7 line (mild phenotype) and the 4-14 or 6-17 line (no phenotype). In this instance, doubly heterozygous animals (~11-12 transgene copies) displayed skin lesions of greater severity than heterozygous animals in the 5-7 line (data not shown). Taken collectively, these data indicated that the appearance and severity of skin lesions in K16 transgenic mice are a function of transgene copy number and are independent of the site of transgene insertion into the mouse genome.

The Expression of the Human K16 Gene is Properly Regulated in the Skin and other Epithelial Tissues of Transgenic Mice

To examine the tissue distribution of the K16 transgene product, we killed F1 offspring transgenic mice at various times after birth. Sections prepared from various tissues were im-

munolabeled with a polyclonal antiserum raised against, and specific for, a 21-mer peptide corresponding to the predicted sequence of the carboxy terminus of human K16 (see Materials and Methods). Control mouse skin embedded in paraffin did not react with this antiserum (Fig. 2 A). In contrast, nonlesional ventral skin from a 5-7-F1 transgenic mouse showed the expected staining pattern for K16 (Moll et al., 1982a; Stark et al., 1987), in that the epidermis was negative while the outer root sheath of hair follicles was positive (Fig. 2 B). Specialized epidermis known to constitutively express K16, such as that of foot pads (Moll et al., 1982a), showed a faint immunostaining for the transgene product (data not shown). In mildly lesional skin of phenotype transgenic animals, the thickened portions of the epidermis showed a strong signal, in addition to hair follicle profiles (Fig. 2 D). The K16 labeling in thickened epidermis and outer root sheath was mostly restricted to the postmitotic, suprabasal layers, i.e., as previously described for

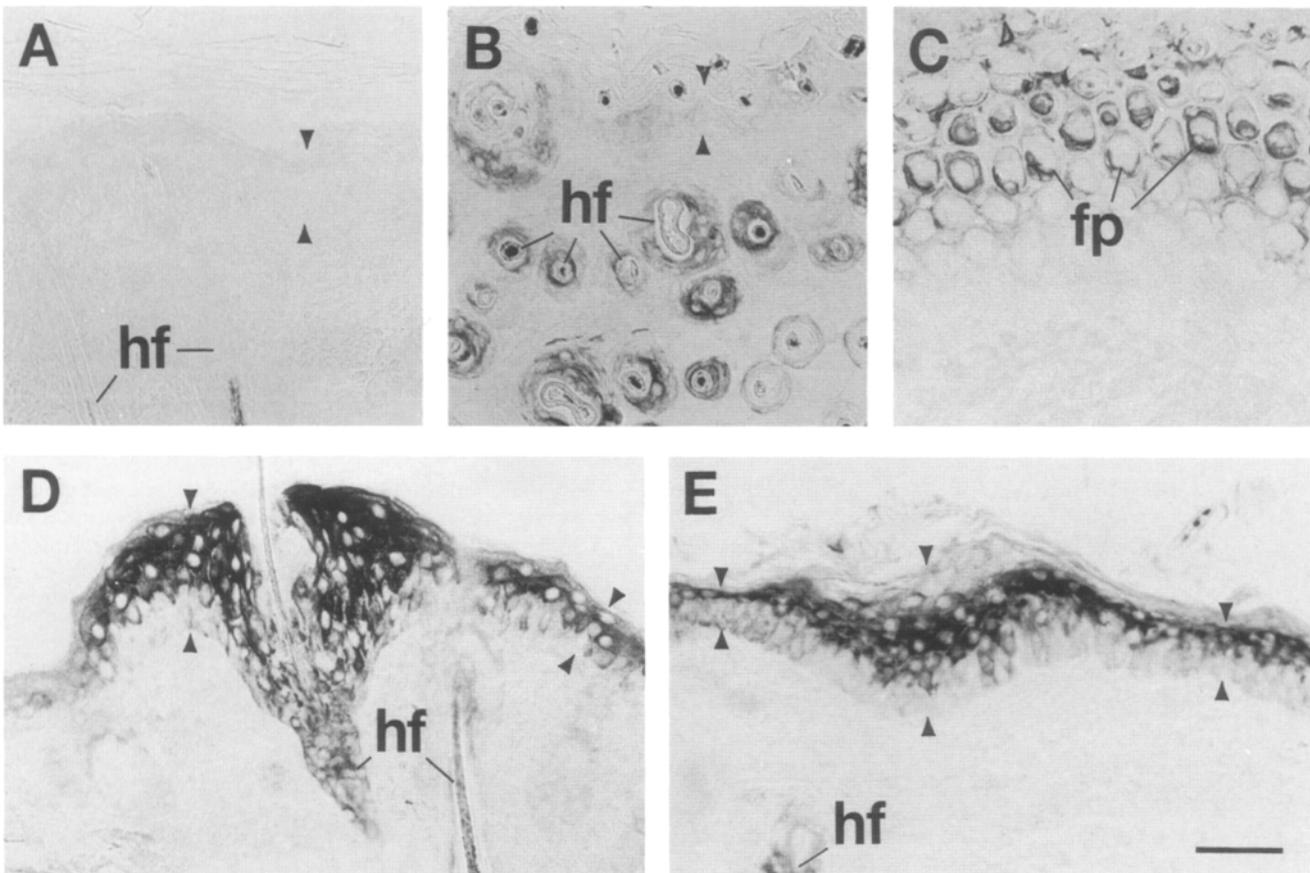


Figure 2. The human K16 transgene is properly regulated in the skin of transgenic mice. 5- μ m paraffin sections were prepared from mouse tissues and subjected to immunostaining using the immunogold-silver enhancement procedure. Sections shown in A-D were stained with a rabbit polyclonal antiserum to human K16, whereas E shows a section stained with monoclonal antibody K8.12. (A) Cross-section of nontransgenic mouse epidermis. Note the complete absence of signal in epidermis (opposing arrowheads) and hair follicle (hf) profiles (the darker band at the bottom corresponds to the natural pigmentation of a hair shaft). (B) Cross-section of nonlesional transgenic mouse epidermis (5-7-F1 transgenic animal). Note the absence of signal in epidermis (opposing arrowheads), while the outer root sheath epithelium of hair follicle profiles are stained. (C) Tangential section of tongue epithelium in a 5-7-F1 transgenic animal. Note that the signal is restricted to filiform papillae (fp). (D) Mildly lesional skin in a 5-7-F2 homozygous transgenic animal. Note the strong signal for the transgene product in the thickened portion of the epidermis immediately adjacent to the outer root sheath of the hair follicle profile. The signal is present in suprabasal layers, while the basal layer is negative. (E) Mildly lesional skin in transgenic founder 4-12. Note the presence of suprabasal staining in thickened epidermis. Omission of the primary antiserum, as well as its preadsorption with the antigen, confirmed the specificity of the signal (data not shown). Opposing arrowheads depict the thickness of the living layers of epidermis. Bar, 50 μ m.

hyperproliferative human epidermis (Stoler et al., 1988; Kopan and Fuchs, 1989a). Identical results were obtained when similar sections were stained with monoclonal antibody K8.12 (Fig. 2 E), which under our labeling conditions reacts only with K16. These data indicate that in the skin of adult transgenic mice with low-to-moderate transgene copy numbers, the human K16 gene is regulated in a tissue- and differentiation-specific fashion, both in its constitutive and inducible components.

Examination of other epithelial tissue provided additional evidence that the human K16 transgene was properly regulated in transgenic mice. In dorsal tongue epithelium, only the filiform papillae were immunostained (Fig. 2 C). Suprabasal staining was also detected in ventral tongue epithelium, palatal epithelium of the oral mucosa, and in esophagus (Moll et al., 1982a). In contrast, glandular epithelia of the skin (sweat and sebaceous glands) and oral mucosa (salivary glands), as well as the tracheobronchial epithelium, were negative for human K16. Simple epithelial tissues such as the liver, lung, intestine, and pancreas, as well

as all nonepithelial tissues examined, were also negative for the transgene product (data not shown), as expected (Moll et al., 1982a).

The Development and Severity of Skin Lesions Correlate with the Levels of Transgene Product in Transgenic Mice

To quantitate the amounts of K16 transgene product present in lesional skin of transgenic mice, we isolated IF proteins from control mouse skin and from nonlesional and lesional transgenic mouse skin, and subjected them to electrophoretic and immunoblot analyses. The results are reported in Fig. 3; A, B and C show immunoblots reacted with anti-human K16, anti-mouse K6, and AE1, a monoclonal antibody reacting with several epidermal type I keratins, while D shows a Coomassie blue-stained gel.

Control mouse trunk skin IF proteins, normal (Fig. 3 A, lane 1) or wounded (Fig. 3 A, lane 2), did not react with the anti-human K16 antiserum under normal IF proteins loading conditions. A faint immunoreaction with a ~49 kD antigen was detected, however, when significantly higher amounts of IF proteins from nontransgenic, wounded skin were analyzed (data not shown). This result, together with additional data referred to below, suggest that this immunoreactive product is the hitherto uncharacterized mouse K16. On the other hand, IF extracts prepared from lesional skin of phenotypic transgenic mice showed readily detectable levels of K16 transgene product (Fig. 3 A, lanes 5-7). Thus, lesional tail skin from a 5-7-F1 heterozygous transgenic mouse (lane 5), lesional ventral skin from a 5-7-F2 homozygous transgenic mouse (lane 6), and lesional ventral skin from founder 4-12 mouse (lane 7) showed markedly elevated levels of the 48-kD transgene product. These lesional samples also showed an elevated level of K6 (Fig. 3 B, lanes 5-7) and elevated levels of the type I keratin 17 (see dots in Fig. 3 D, lanes 5-7). In most but not all extracts prepared from lesional skin, a concomitant decrease in the levels of the differentiation-specific K10 occurred (Fig. 3 C, lanes 5-7). Differences between extracts may be partially caused by inclusion of variable amounts of healthy tissues during the sampling procedure. Such changes in the keratin profile of skin are typical of the hyperproliferation ensuing wounding (Fig. 3, B-D, lane 2), or those associated with several diseases (Sun et al., 1984; Stoler et al., 1988). When equivalent amounts of IF proteins from nonlesional transgenic skins were analyzed, however, no transgene product could be detected (Fig. 3 A, lanes 3 and 4). In fact, higher amounts of skin IF extracts (3×) had to be loaded to detect the 48-kD transgene product in these samples (data not shown). These data are consistent with the immunohistochemical data (Fig. 2) showing that K16 is constitutively expressed in the outer root sheath (ORS) of hair follicles, but is strongly induced in ORS and epidermis undergoing hyperproliferation or abnormal differentiation.

Given that mouse K16 has yet to be isolated and characterized (Schweizer, 1993), mouse K17 was used as a reference to estimate relative transgene protein levels. The expression of K17 is coregulated with K16 in human skin (Moll et al., 1982a, 1982b), and in fact, is more abundantly induced when human epidermal keratinocytes are cultured in vitro, a context mimicking hyperproliferation (e.g., Weiss et al.,

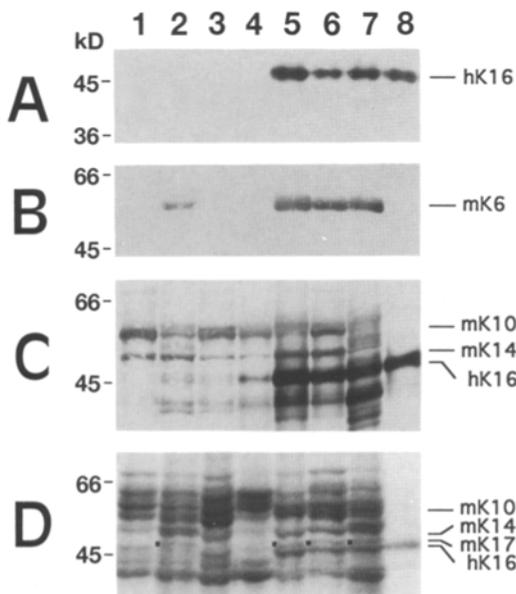


Figure 3. Estimation of the levels of human K16 in transgenic mouse skin. Intermediate filament proteins were extracted from skin, electrophoresed using SDS-PAGE, and either electroblotted onto nitrocellulose or stained with Coomassie blue. Loadings are as follows: lane 1, control (nontransgenic) mouse skin; lane 2, nontransgenic mouse skin 72 h after multiple incisional wounding; lane 3, nonlesional trunk skin from 4-14-F1 transgenic mouse; lane 4, nonlesional trunk skin from 5-7-F1 transgenic mouse; lane 5, lesional tail skin from 5-7-F1 transgenic mouse; lane 6, lesional trunk skin from 5-7-F2 homozygous transgenic mouse; lane 7, lesional trunk skin from 4-12 transgenic founder mouse; lane 8, 100 ng of FPLC-purified human K16. (A) Blot (1.5 μ g protein/lane) incubated with a rabbit polyclonal anti-human K16. (B) Blot (3 μ g protein/lane) incubated with a rabbit polyclonal anti-mouse K6. (C) Blot (1.5 μ g protein/lane) incubated with monoclonal antiserum AE1, which recognizes several type I epidermal keratins. (D) Coomassie blue-stained gel (15 μ g proteins/lane). The migration position of molecular mass markers is indicated on the left, and the position of selected type I keratins is indicated on the right (see Schweizer, 1993). Mouse K17, a 50-kD protein comigrating with purified recombinant human K14 (not shown), is depicted by dots in D.

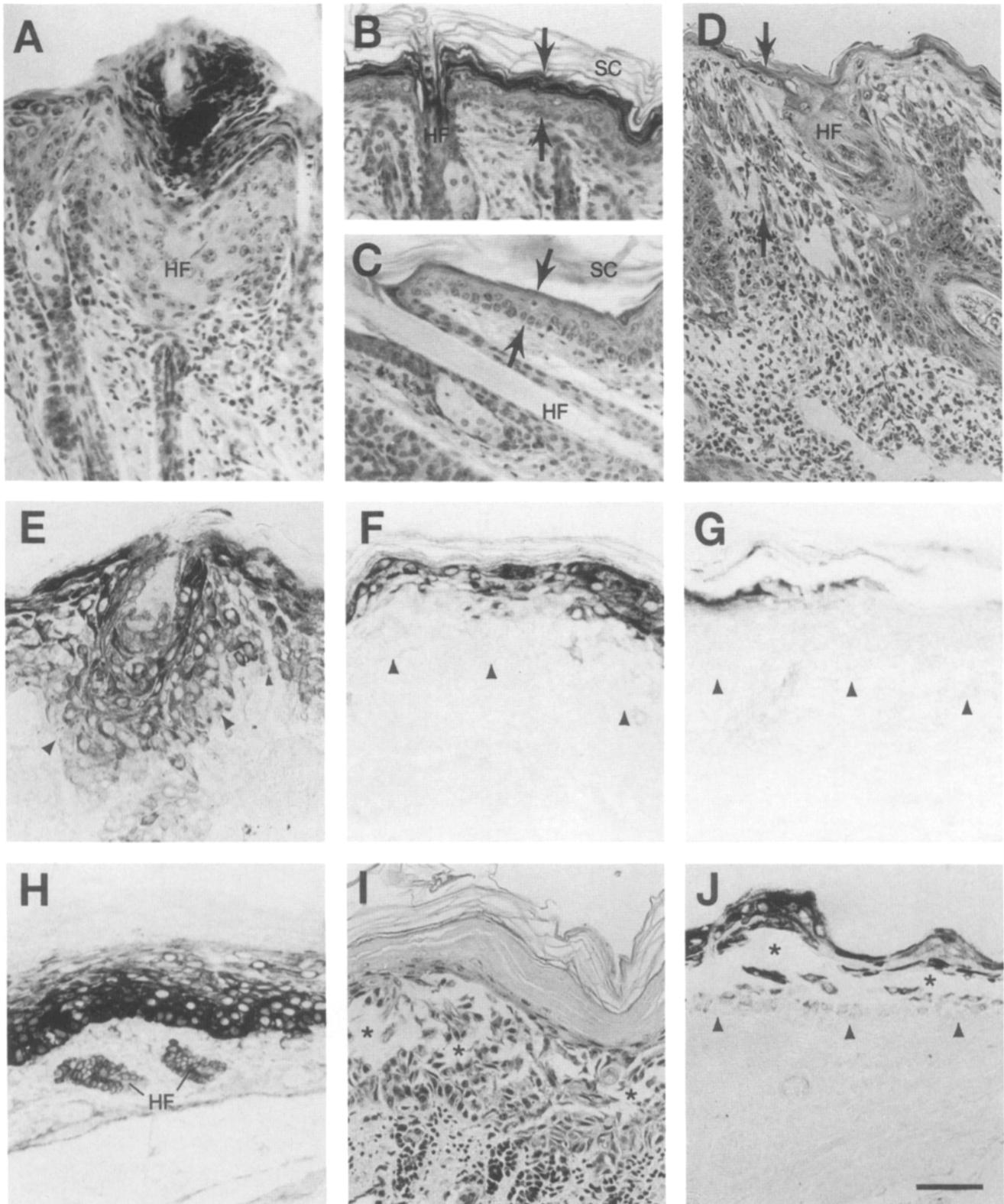


Figure 4. Light microscopy and immunohistochemistry of lesional skin in phenotypic K16-transgenic mice. (A–D and I) Hematoxylin-eosin-stained paraffin sections, in which the thickness of the living epidermal layers is indicated by opposing arrows. (E–H and J) Paraffin sections subjected to immunogold-silver enhancement staining procedure; the position of the basement membrane zone is indicated by arrowheads. All micrographs shown are at the same magnification. (A) Follicular parakeratosis in trunk skin of an 11-d-old mildly phenotypic transgenic mouse. Compare with B, which shows a section from corresponding area in a nontransgenic mouse. (C) Tail skin from a 14-d-old control mouse. (D) Severely affected tail skin from a 14-d-old phenotypic transgenic mouse. In addition to pronounced thickening of the epidermis and inflammatory changes in the underlying dermis, note the intraepidermal blistering caused by the disruption of cell-cell

1984; Korge et al., 1990). The cloned mouse K17 cDNA (Knapp et al., 1987) encodes a 50-kD protein that can easily be recognized in IF extracts prepared from regenerating control mouse skin (Fig. 3 D, lane 2) or from lesional transgenic mouse skin (lanes 5–7). Judging from the Coomassie blue staining, lesional transgenic mouse skin contains two to three times as much human wild-type K16 protein as mouse K17. On a skin tissue basis, thus, “foreign” K16 represents a minor fraction of total keratins (<5–7%), even in lesional skin.

Follicular and Epidermal Dyskeratosis and Acantholysis are the Major Histopathological Changes in Lesional Skin of Transgenic Mice

To determine the nature of the skin lesions and their relationship to the expression of the transgene, we conducted light microscopy and immunohistochemical analyses before and after their inception in phenotypic mice. The initial pathological change seen in lesional transgenic skin consisted of a thickening of the ORS in the upper portion of hair follicles and of proximal epidermis, along with anomalies in terminal differentiation typical of hyperkeratosis and dyskeratosis (Fig. 4 A; a control is shown in B). The architecture of these two epithelia was perturbed, as evidenced by significant expansions of the spinous layer (acanthosis, partly caused by keratinocyte hypertrophy) and stratum corneum layer (hyperkeratosis). Aberrancies in the granular layer, including its disappearance (agranulosis), as well as occasional hypergranulosis, were also noted (Fig. 4 A). In more severely affected transgenic skin, the follicular changes were more pronounced and involved larger portions of interfollicular epidermis (Fig. 4 D; see corresponding control in C). In particular, the frequent occurrence of large gaps between suprabasal keratinocytes in the perifollicular area suggested an impairment of cell–cell adhesion (see acantholysis in Fig. 4 D). Moreover, the stratum corneum layer was often further thickened and disorganized. No signs of cytolysis could be detected. Such lesions were seen in two independent instances, e.g., in transgenic founder 4–12, and in heterozygous and homozygous mice derived from founder 5–7. In contrast, they were not seen in control mouse skin (Fig. 4, B and C), or in nonphenotypic transgenic mouse skin (data not shown).

Immunohistochemistry indicated that in all cases, mild and severe lesional skin displayed a strong signal for the K16 transgene product, and as expected, this signal was mostly found in the suprabasal layers of ORS and proximal epidermis (Fig. 4 E). Immunolocalization of additional antigens further confirmed that the program of terminal differentiation was abnormal. First, a strong suprabasal staining for K6 was detected in lesional transgenic epidermis (data not

shown). Second, K10, an early marker of terminal differentiation, was present only in the uppermost suprabasal layers (Fig. 4 F), and in some cases, was completely absent. Third, filaggrin, a late marker of terminal differentiation, was often decreased or even absent compared to nonlesional epidermis (Fig. 4 G). Finally, K14 localization was typical of hyperproliferative epidermis (see Coulombe et al., 1991a), in that in addition to the basal layer, the signal extended well into the suprabasal compartment (Fig. 4 H). That mitotic activity was enhanced in lesional epidermis was confirmed using BrdU incorporation followed by immunohistochemistry with an anti-BrdU antibody (data not shown).

In a subset of 5-7-F2 homozygous transgenic animals (~16–20 transgene copies), similar skin lesions appeared between day 2 and 5 after birth, before hair follicle maturation had been completed. Light microscopy of such lesions revealed similar alterations to those described above, with occasional blistering within the suprabasal compartment of epidermis (Fig. 4 I). When immunostained with the anti-human K16 antiserum, these lesions showed a strong suprabasal staining (Fig. 4 J). In these homozygous animals, in fact, the epidermis was positive for the transgene product as soon as day 1 after birth, i.e., before lesions appeared (data not shown). We noticed that several 5-7-F2 homozygous transgenic offsprings were significantly smaller than heterozygous and nontransgenic littermates, and they featured epithelial thickening and dyskeratosis in portions of the oral mucosa. Founder 4-12, on the other hand, died at 2 mo of age and had rather severe skin lesions throughout most of its body. Histological survey of a number of epithelial tissues, however, failed to reveal the cause of death in the most severely phenotypic transgenic animals.

Cytoarchitectural Aberrations and Deficiency in Keratinocyte Cell–Cell Adhesion in Lesional Skin of Phenotypic Transgenic Mice

To examine the skin lesions at higher resolution, conventional and immunogold electron microscopy studies were conducted in several phenotypic transgenic mice and in age-matched control mice. Skin was sampled before, as the lesions developed, and after the lesions had become severe. Our analyses emphasized the junctional area between the hair follicle ORS and epidermis. The first detectable alteration was a disorganization of keratin filament network in spinous keratinocytes, and this preceded the onset of a phenotype (Fig. 5, A vs B, transgenic and control, respectively). Disrupted keratin filaments were often collapsed near or around the nucleus (A). In phenotypic skin, a disorganization of keratin filaments was also noted in granular keratinocytes (data not shown), and was accompanied by other alterations in cytoarchitecture. Indeed, keratinocytes

contacts in the suprabasal layers of epidermis. (E) Anti-human K16 staining of thickened outer root sheath and proximal epidermis in phenotypic transgenic mouse. Note the abundance of transgene product in suprabasal layers of these epithelia. (F) Anti-K10,K11 staining of thickened epidermis in phenotypic transgenic mouse. The signal is only present in the uppermost layers of the epidermis. (G) Anti-filaggrin staining of thickened epidermis in phenotypic transgenic mouse. Note the complete absence of signal on the right side of the epidermis shown on this field. (H) Anti-K14 staining of lesional epidermis in phenotypic transgenic mouse. The signal extends from the basal layer to the middle of the suprabasal layers. (I and J) Acantholytic epidermis in 2-d-old phenotypic transgenic mouse. I shows a hematoxylin-eosin-stained section, while J shows a similar lesion immunostained with anti-human K16. Note that the acantholytic cells display a strong signal for the transgene product. HF, hair follicle; SC, stratum corneum. Asterisks denote intraepidermal blister formation. Bar, 50 μ m.

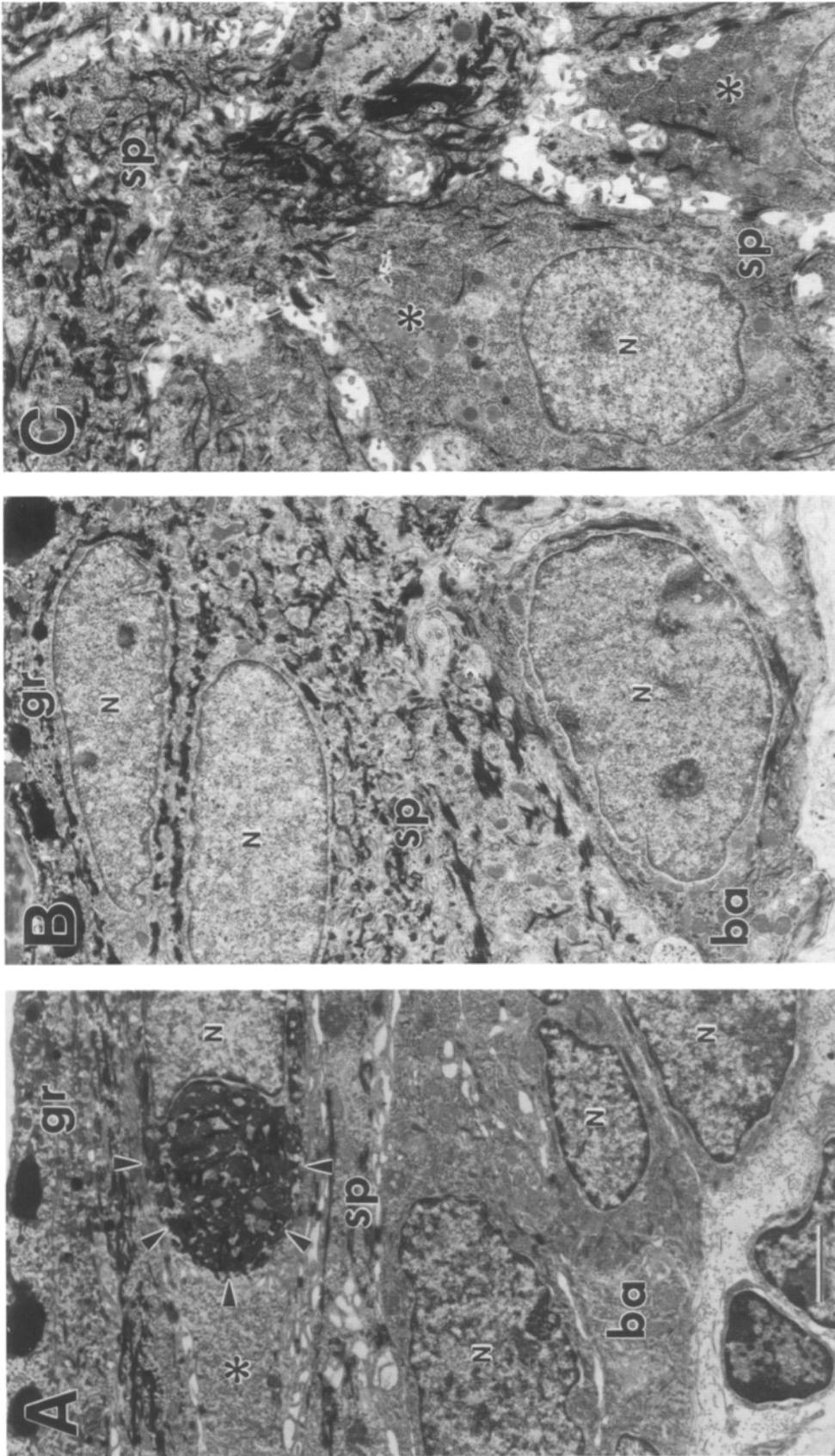


Figure 5. Electron microscopy of tissue alterations in epidermis of phenotypic K16-transgenic mice. Low magnification electron microscopy survey of the epidermis in (A) transgenic mouse epidermis before the development of lesion, (B) control (nontransgenic) mouse epidermis, and (C) transgenic mouse epidermis characterized by moderate dyskeratosis. Note that all three micrographs are at same magnification (bar, 1 μ m), and that in A and B, the basal (ba), spinous (sp), and granular (gr) layers of epidermis (collectively known as the living compartment) are comprised within that space. In contrast, in established lesions such as that shown in C, only a portion of the much enlarged spinous layer can be seen at the same magnification. (A) Early phase of the reorganization of the keratin filaments in transgenic epidermis. The arrowheads outline the periphery of tonofilaments that are collapsed by the nucleus (N) in a spinous layer keratinocyte, a process that depletes portions of the cytoplasm of its normal keratin content (*asterisk*). At this early stage, the architecture of the epidermis is otherwise normal. (B) Control mouse epidermis. Note the complete reorientation of the keratinocyte main axis in the spinous layer, the flattened shape of suprabasal cells (sp and gr), the homogeneous distribution of the tonofilament profiles in the cytoplasm, and the tight adhesion between cells. (C) Spinous layer in moderately lesional epidermis of a phenotypic transgenic mouse. Micrograph orientation is identical to A and C, with the skin surface located upward. Keratinocyte cell shape and cell axis with respect to tissue architecture are significantly altered. Note the occurrence of large areas of cytoplasm devoid of tonofilaments (*asterisks*), as well as the widening of intercellular spaces between keratinocytes.

failed to flatten parallel to the skin surface as they normally do, and instead, they presented a polygonal shape and the overall morphology of an "activated" cell, i.e., they featured numerous surface projections and a reduced density of desmosomes, correlating with a significant widening of intercellular space between keratinocytes (Fig. 5 C). In severely dyskeratotic cells, the nucleus often had an aberrant shape (lobated/indented; data not shown). These changes were never seen in control mouse skin (Fig. 5 B) or in nonphenotypic transgenic mouse skin (data not shown).

Examination of these lesions at higher magnification revealed additional features of significant interest. Thus, small protein aggregates were present in the cytoplasm of a subset of keratinocytes in lesional ORS and epidermis. The aggregates were seen initially in spinous keratinocytes, and their presence correlated with a reorganization of keratin filaments and preceded the development of a skin phenotype (Fig. 6 A). In established lesions, the number of keratinocytes featuring aggregates and the number of aggregates per cell was roughly proportional to the dyskeratotic changes at the tissue level. In many instances, these aggregates were intermingled with randomly oriented and disorganized tonofilament bundles near the nucleus (Fig. 6, A vs B). A fraction of basal cells contained these cytoplasmic aggregates (data not shown), consistent with the occurrence of K16-immunopositive basal cells as seen by immunohistochemistry, and the known existence of postmitotic keratinocytes in the basal layer (Schweizer et al., 1984). To ascertain the protein composition of these aggregates, we performed colloidal gold immunoelectron microscopy. As expected, all aggregates stained intensely with the antiserum against the K16 transgene product. K16 labeling was also detected in keratin filament bundles in the suprabasal layers of thickened epidermis, confirming that the transgene product had been recruited for filament assembly (Fig. 6 C). These aggregates also contained K14, as evidenced by gold decoration using an antiserum to this keratin (Fig. 6 C, inset). This suggested that the transgene product had successfully "displaced" a fraction of the K14 pool that was paired to type II keratins. Unexpectedly, a limited number of small K16-positive aggregates were seen in the nucleus of severely dyskeratotic granular cells in a subset of phenotypic transgenic mice (Fig. 6 D). Given that (a) not all lesional transgenic skin samples showed these nuclear aggregates; (b) the pathogenic process was initiated at an earlier stage of differentiation, i.e., in the lower spinous layer; and (c) these nuclei were otherwise normal, we believe it is highly unlikely that intranuclear accumulation of the transgene product played a significant role in eliciting the phenotype (for a discussion of the occurrence of wild-type keratins in the nucleus, see Blessing et al., 1993).

Remarkable changes also characterized desmosomes and mitochondria in lesional transgenic skin. Thus, the peripheral cytoplasm of suprabasal keratinocytes in lesional transgenic skin often appeared "depleted" of tonofilaments, and this correlated with a reduced density of desmosomes at the cell surface (Fig. 6, E vs B, control). In addition to this, two major types of anomalies were noted in the ultrastructure of desmosomal plaques: a conspicuous absence of tonofilament anchorage onto the cytoplasmic side of the plaque, and less frequently, the complete "halving" of the plaque at the level of its intercellular portion (*disadhesion*; Fig. 6, D-F). Such loss in desmosome integrity was often associated with de-

crease in keratinocyte cell-cell adhesion (*acantholysis*; see Fig. 4 I), and was more prominent in the spinous layer compared to the granular layer. Alterations in the distribution and ultrastructure of mitochondria also occurred in lesional transgenic ORS and epidermis. In mildly phenotypic skin, one or two electron-dense spherical inclusions were present in some mitochondria of suprabasal cells (Fig. 6 F). The intracellular distribution and ultrastructure of mitochondria appeared otherwise normal. In severely dyskeratotic skin lesions, however, mitochondria were often clustered and enlarged, and contained up to four of these inclusions in their matrix (Fig. 6 F, inset), albeit the keratinocytes featuring them were clearly not degenerating. Such alterations in desmosomes and mitochondria were never seen in control mouse skin (e.g., Figs. 5 C and 6 B) or in nonphenotypic transgenic mouse skin.

Probing the Relationship between Keratin Expression and Desmosomal Alterations in Lesional Transgenic Mouse Skin

Taken together, our immunohistochemical and ultrastructural analyses in lesional transgenic outer root sheath and epidermis implied the existence of a complex relationship between (a) aberrant keratinization; (b) the intracellular levels of the type I keratins K16 and K10, and (c) desmosomal alterations. To probe the nature of this relationship further, we performed double-immunofluorescence labeling experiments using anti-K16, anti-K10/K11, and anti-desmoplakin I antisera on fresh frozen sections prepared from ventral skin lesions of moderate severity. Desmoplakin I is a major structural component of desmosomes (Jones and Green, 1991). Representative results are shown on Fig. 7; A shows the results of double labeling for K16 and K10/K11. In epidermis showing a near-normal thickness and architecture, the onset of K10/K11 expression clearly precedes and appears to exceed that of K16. Conversely, in thickened epidermis, expression of K16 precedes that of K10/K11, and all three keratins are present in the upper suprabasal layers (Fig. 7 A). These results are in good agreement with the single-labeling experiments reported in Fig. 4 and the protein data reported in Fig. 3. Fig. 7, B-E, show the results of double-labeling for K10 and desmoplakin I. In epidermis showing a normal architecture and normal distribution of K10, the desmoplakin signal is also normal, in that it is located at the cell-cell interface throughout the epidermis (Fig. 7, B and C) and the outer root sheath of hair follicles (not shown). In striking contrast, thickened and disorganized epidermis is characterized by an almost complete absence of K10 signal, and it also shows aberrancies in the distribution of desmoplakin I (Fig. 7, D and E). Indeed, this latter appears discontinuous and/or irregular in some areas (Fig. 7, D and E). These data strongly suggest that in lesional transgenic mouse epidermis, desmosomal alterations were not solely a function of abundant K16 expression, but also of a reduced expression of K10 and, possibly, of other differentiation-specific keratins.

Discussion

Modest Overexpression of Intact Human K16 Results in Skin Lesions in Transgenic Mice

In this study, we have introduced the intact human K16 gene

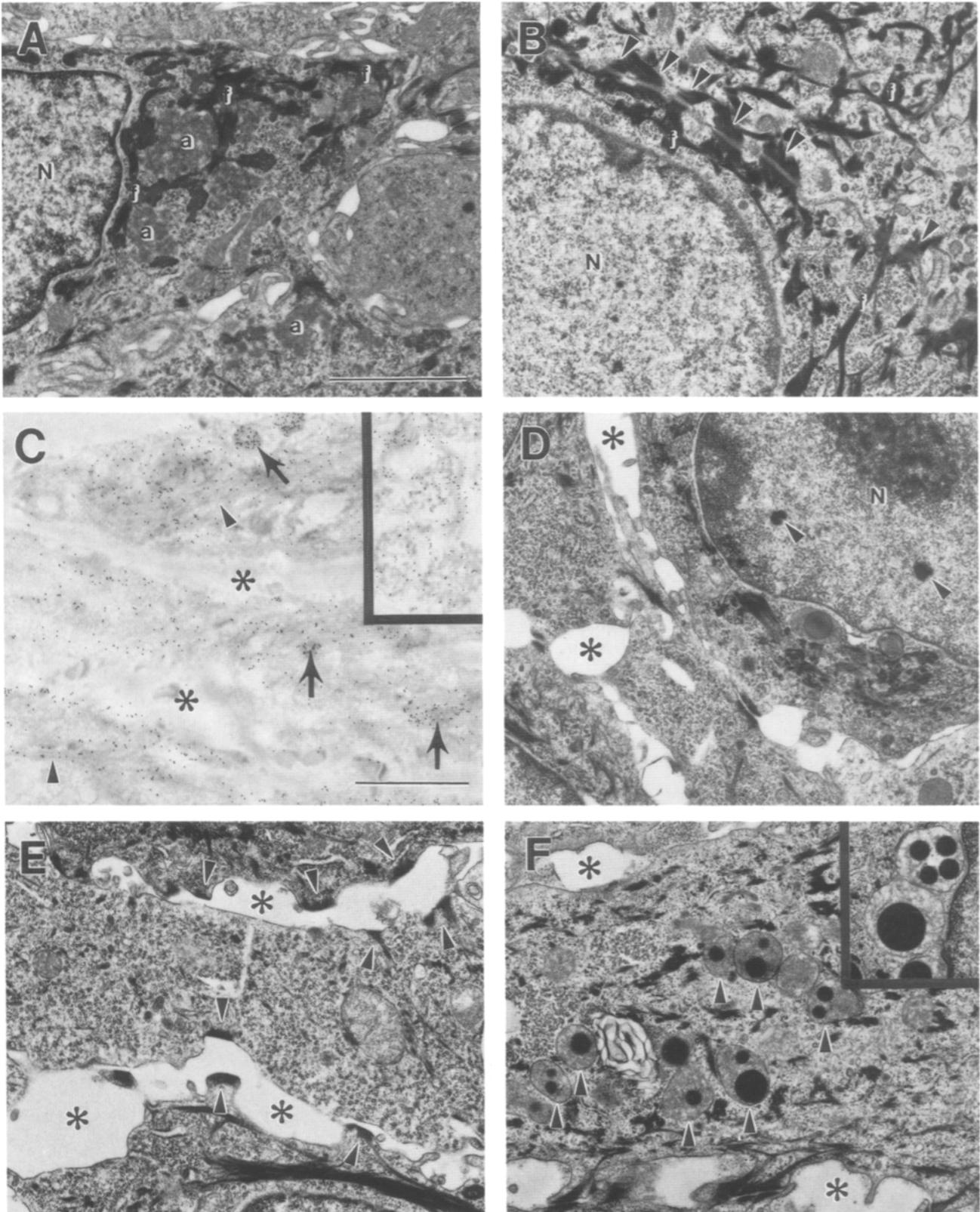


Figure 6. Electron microscopy of cellular alterations in epidermis of phenotypic K16-transgenic mice. (A, B, D, E, and F) Conventional transmission electron microscopy. (C) Immunogold electron microscopy. (A) Presence of protein aggregates (a) intermingled with keratin filaments (f) in the cytoplasm of a spinous layer keratinocyte in mildly dyskeratotic transgenic mouse epidermis. This contrasts with the ultrastructure of spinous cells in control mouse epidermis shown in B. No such aggregates are seen, and the keratin filaments (f) are organized radially in the cytoplasm, and are anchored at the level of desmosomes at the cell surface (arrowheads). (C) Immunogold staining of acantholytic transgenic epidermis with an anti-human K16 antiserum. The keratin filament bundles are decorated by gold particles (ar-

in the germline of transgenic mice and demonstrated that in the skin, particularly, and in several other epithelial tissues surveyed, it is regulated in a tissue-specific (human K16-like) fashion. Transgenic mouse lines with low transgene copy numbers and low levels of expression did not show any detectable alteration in the relevant skin epithelial tissues. In striking contrast, however, transgenic mice with higher transgene copy numbers and more substantial transgene expression were characterized by skin lesions consisting in papules, crusty erosions, and scaling. Skin lesions were more prominent in preferential body sites. Obvious sites of predilection included intertriginous areas (regions showing skin folds), tail skin, and in the more severely affected animals, the ventral body surface. The reasons underlying the relative specificity of the lesional process are currently unknown.

Morphologically, we have shown that in such transgenic animals, the lesional process began in the upper portion of hair follicle ORS, where the transgene is known to be constitutively expressed (Moll et al., 1982a; Stark et al., 1987). Given that the epidermis is continuous with the ORS, and that various types of conditions, including hyperproliferation, are associated with the induction of K16 expression in the epidermis proper (Weiss et al., 1984; Stoler et al., 1988), transgene expression did occur in the epidermis proximal to morphologically abnormal ORS. The distribution of several markers of the program of terminal differentiation in affected epidermis was abnormal in phenotypic transgenic mice, correlating with profound changes in the architecture of the tissue, as well as the occurrence of a pronounced hyperkeratosis (thickening of stratum corneum layer). Our analyses provided strong evidence that after its initiation in ORS, the pathogenic process progressed according to the extent of K16 expression, i.e., its severity was determined by transgene copy number and transcriptional activity in each of these copies. This interpretation is supported by the morphological and biochemical data accumulated in our various transgenic mouse lineages.

In at least one subset of transgenic mice (homozygous offspring derived from 5-7 founder), skin lesions appeared within days after birth, well before hair follicle morphogenesis had been completed (see Kopan and Fuchs, 1989b). Immunohistochemical studies revealed the presence of the transgene product in epidermis in 1-d-old pups, i.e., before the skin lesions develop between 2 and 4 d after birth. While the K6, K16, and K17 genes are expressed transiently in human epidermis during fetal development, the adult keratin pattern is already established in newborn epidermis and thus excludes these keratins (Moll et al., 1982b). The human K16 gene has been reported to be regulated at the transcriptional

and posttranscriptional levels in the epidermis (Tyner and Fuchs, 1986). Therefore, it is conceivable that a nuclear or a cytoplasmic factor involved in the repression of the K16 gene or mRNA, respectively, was being titrated out in these high transgene copy number mice, thus leading to inappropriate (ectopic) expression. Further characterization of these mice should allow us to distinguish among these and other possibilities.

An important question left unresolved is whether the phenotype in transgenic mice resulted from an increased expression of a truly wild-type K16 sequence, or alternatively, whether the human K16 sequence is perceived as a "mutant" by mouse epithelial cells. There is confusion in the literature regarding mouse K16 which, as it turns out, has yet to be characterized (see Schweizer, 1993). Examination of epidermal keratin orthologues in mice and humans reveals very high sequence identity scores, increasing the likelihood that these sequences are functionally interchangeable. Consistent with this, epithelial lesions were not described in previous studies in which human K14 (Vassar et al., 1989), human K18 (Abe and Oshima, 1990), human K1 (Rosenthal et al., 1991), and bovine K19 (Bader and Franke, 1990) were "overexpressed" in a tissue-specific fashion in transgenic mice. One exception to this is the brittle hair phenotype generated by massive overexpression (~ 250 copies) of a sheep hair-specific type II keratin gene in the mouse (Powell and Rogers, 1990). Before we can formally attribute the phenotype observed in transgenic mice to the increased expression of K16, a control experiment in which the expression of K14 or K10 is driven to comparable levels in transgenic mouse skin is needed.

On the other hand, the tissue-specific expression of a dominant negative mutant engineered in the human type I K10 sequence resulted in the trauma-induced lysis of keratinocytes in the spinous layer of transgenic mouse epidermis (Fuchs et al., 1992). The mouse phenotype produced closely resembled EHK, a genetic skin disease caused by point mutations in either one of the K1, K9, or K10 genes (reviewed by Coulombe and Fuchs, 1994). As expected, cytolysis of postmitotic epidermal cells stimulates mitotic activity in the basal layer, and probably accounts for the chronic hyperproliferation, significant thickening of the epidermis, and hyperkeratosis typical of this disorder (Fuchs et al., 1992). The comparison of these results with those obtained in our studies is relevant because after its expression is induced in epidermis, the K16 transgene is regulated like a K10 gene. Quite remarkably, a surprising degree of hyperproliferation, acanthosis (epidermal thickening), and hyperkeratosis, occurred in the absence of detectable cytolysis in our phenotypic transgenic mice. Fuchs et al. (1992) suggested the

rowheads), as are nonfilamentous protein aggregates (*arrows*). These aggregates are also decorated with gold particles when an anti-K14 antiserum is used (*inset*). Note also the presence of large gaps between keratinocytes (*asterisks*). (*D*) Illustration of the occasional presence of small protein aggregates in the nucleoplasm of a subset of transgenic keratinocytes (*arrowheads*). Immunoelectron microscopy confirmed that these nonfilamentous aggregates contain the transgene product (not shown). The ultrastructure of the nucleus appears normal. Again, note the relative paucity of desmosomes at the cell surface and the wide gaps between cells. (*E*) Desmosomal alterations in spinous keratinocytes of phenotypic transgenic mouse epidermis. The desmosomes, or their remnants, are depicted by arrowheads. In many instances, no keratin filaments are attached onto the cytoplasmic side of the plaque, and several plaques are separated at the level of the intercellular domain, causing gaps between keratinocytes (*asterisks*). Compare these altered desmosomes with those depicted in *B*. (*F*) Mitochondrial alterations in the suprabasal keratinocytes of phenotypic transgenic mouse skin. One or several spherical and electron-dense inclusions are present in the mitochondrial matrix, and several of these mitochondria are clustered. The inset shows mitochondria that are enlarged and present an unusually electron-lucent matrix. N, nucleus. Bars, 2 μm . (Bar in *A* also applies to *B*, *D*, and *E*).

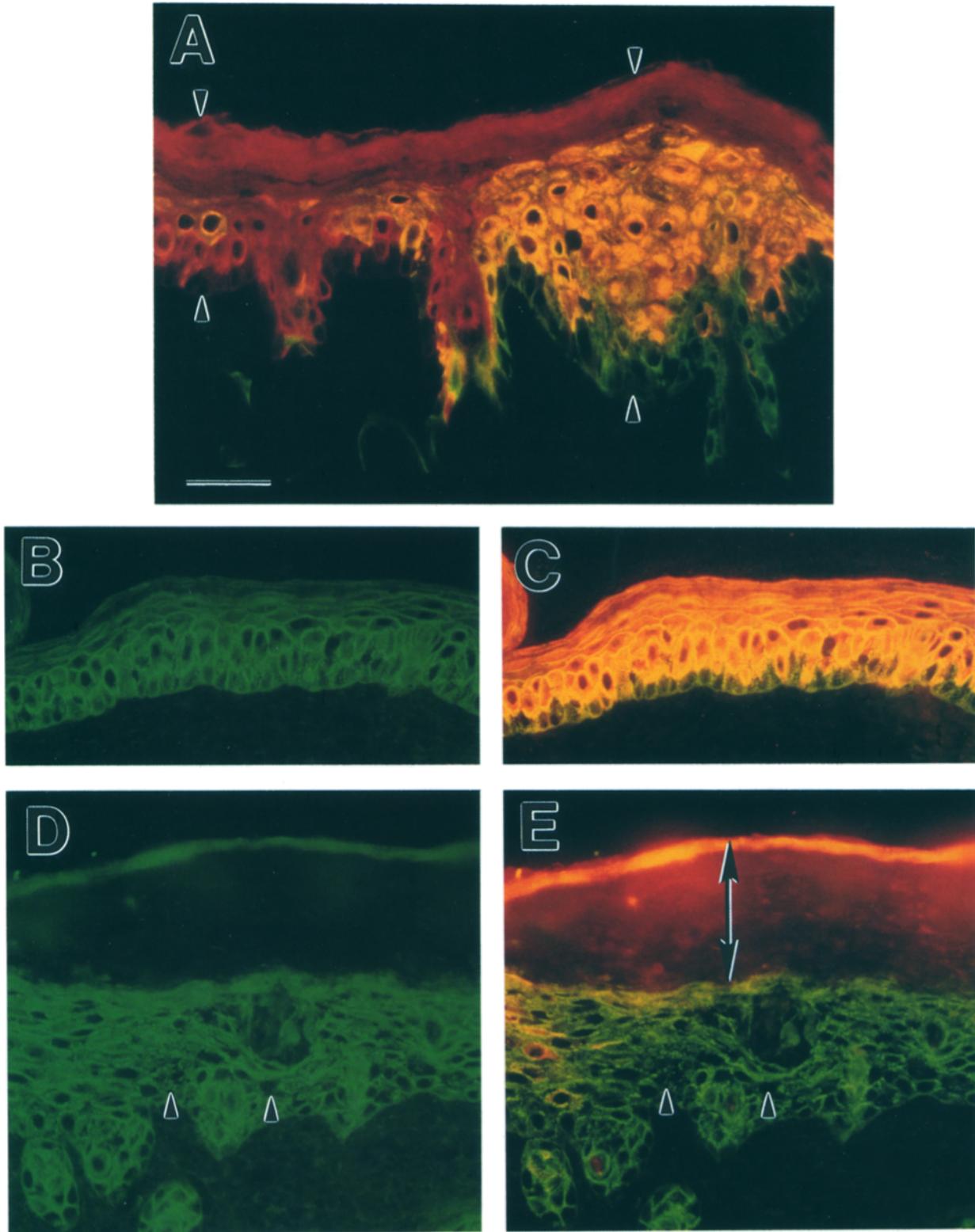


Figure 7. Double-immunofluorescence labelings in moderately lesional transgenic mouse ventral skin. 5–7- μ m sections were prepared from freshly frozen ventral skin sampled from a transgenic mouse with a phenotype of moderate severity, and were processed for double-immunofluorescence labeling as described in Materials and Methods. (A) Superposed immunofluorescence signals for K16 (FITC channel, green) and K10/K11 (Texas red channel, red). The thickness of the epidermis is depicted by opposing arrowheads. Note that in the thinner epidermis at left, the K10/K11 signal clearly predominates over the K16 signal. In contrast, in the thickened epidermis on the right, the first layer of suprabasal cells expresses only K16 because it is only FITC positive. The remainder of the suprabasal layers are yellow-orange: this color is the result of superposition of the signals for K16 (green) and K10/K11 (red), and it indicates that both keratins are present. (B–E) Double immunofluorescence for desmoplakin I (FITC channel, green) and K10/K11 (Texas red channel, red). (B) The signal for

existence of pathways of communication between the proliferating and differentiating compartment in epidermis; our findings further extend this, and they indicate that these pathways could be exquisitely sensitive to alterations in the normal program of terminal differentiation.

Increased K16 Expression in Transgenic Mouse Skin Results in Remarkable Cellular Alterations

Our results clearly established that at the cellular level, the overexpression of a wild-type human K16 gene in transgenic mouse skin, along with the other alterations in the program of keratin gene expression, is associated with a major reorganization of keratin filaments in postmitotic keratinocytes of the ORS and epidermis. Ultrastructural examination of mild and severe skin lesions at early stages of their development indicated that this reorganization initially consisted of a redistribution of filament bundles near or around the nucleus, thus partially depleting the peripheral cytoplasm of keratinocytes of keratin filaments. Such changes in keratin filament organization preceded (and thus might have been responsible for) the onset of several other types of cellular alterations in transgenic keratinocytes. These alterations included changes in keratinocyte cell shape and cytoarchitecture, in the number and structure of cell surface desmosomes, and of organelles such as mitochondria.

The notion that keratinocyte cytoarchitecture is partly dependent on the organization of the keratin filament network is already established. Indeed, aberrations in nuclear shape and in the spatial distribution of cytoplasmic organelles have been described in EBS and EHK diseases, two keratin gene disorders in which the keratin filament network of basal and suprabasal epidermal cells, respectively, is structurally and functionally defective (Fuchs and Coulombe, 1992). Our results extend these previous findings, and they suggest that the "disruption" of the organization of keratin filaments in postmitotic epidermal keratinocytes caused by plain alterations in the program of keratin gene expression interferes with the normal flattening and reorganization of the cell's main axis known to occur under normal differentiating conditions. In future studies, we will investigate the specific contribution of greatly elevated intracellular levels of K16 (along with K6 and K17) in the causation of such major cytoarchitectural changes in skin keratinocytes, for this would have major implications for wound healing in complex epithelia.

Whether keratin filaments play a direct role in maintaining the structure and function of desmosomes at the cell surface is, on the other hand, still a matter of debate. Transfection of mutant keratin cDNAs in cultured F9 embryonal carcinoma cells (Trevor, 1990) and in SCC13 epidermal cells (Albers and Fuchs, 1989) results in quantitative and/or qualitative anomalies in the distribution of desmosomal antigens. Likewise, expression of desmoplakin mutants in cultured epithelial cells interferes with assembly of desmosomes at the

cell surface and causes aberrations in the organization of keratin filaments (Stappenbeck and Green, 1992). Moreover, there are studies reporting a lower number of desmosomes in lesional epidermis of EBS patients (Ito et al., 1991; McGrath et al., 1992), and in patients suffering from some forms of EHK (e.g., Moriwaki et al., 1988). However, evidence against a major role of keratin IFs in desmosome assembly and function stemmed particularly from the study of homozygous K8 null mouse ES cells. Indeed, when stimulated to undergo embryonic body formation in vitro, these cells are capable of forming a simple polarized epithelium with normal desmosomes in spite of the complete absence of keratin filaments (Baribault et al., 1993). Thus, there may exist multiple pathways for the assembly of functional desmosomes at the cell surface, and their relative importance may vary among epithelial cell types. Our results suggest that at least some types of keratin filaments could play a functional role in the maintenance of functional desmosomes at the cell surface in ORS and epidermis. One can envision several mechanisms through which the composition and structure of keratin filaments could directly or indirectly influence cell-cell adhesion between keratinocytes. One possibility is that filaments enriched in particular keratins could interact with key structural components of desmosomes and influence their normal assembly or function. Alternatively, regulatory components that are directly involved in desmosome assembly and dynamics could be physically associated with such keratin filaments, and thereby depend on IF organization in the cytoplasm. Yet another alternative is that the disruption of the normal organization of keratin filaments could, under some conditions, trigger the production/activation of factors involved in desmosome regulation. Finally, the protein composition of desmosomes undergoes modifications as epidermal cells differentiate (Arnemann et al., 1993), and this "maturation process" may depend on the cytoplasmic organization of keratin filaments. Additional studies will be required to settle among these and other possibilities, and again, to determine the specific contribution of the increased levels of K16, K6, and K17 to the observed effects on desmosomes in our transgenic mice.

Finally, intriguing changes were noticed in the ultrastructure of mitochondria in moderately and severely lesional transgenic skin. Smooth-surfaced, spherical, and electron-dense inclusions of various sizes were present in the mitochondrial matrix. These inclusions do not contain keratin, as indicated by our immunoelectron microscopy studies. Interestingly, similar mitochondrial inclusions have been described in mouse skin subjected to UVB irradiation or tumor promoters, such as TPA or mezerein (see Feldman et al., 1990, and references therein). At the present time, however, we do not know the significance or the metabolic consequences of the occurrence of such mitochondrial alterations in transgenic keratinocytes, which clearly did not show any signs of degeneration.

desmoplakin I in a near-normal area of transgenic epidermis. The signal is concentrated at the cell-cell boundaries. (C) The exact same field, except that the signals for desmoplakin I and K10/K11 are now superposed. Keratins K10/K11 are present throughout the suprabasal layers, and the boundaries between suprabasal keratinocytes is intensely yellow. (D) The desmoplakin I signal in a lesional area of transgenic mouse epidermis. Note that in several areas the signal is irregular and discontinuous (*arrowheads*) compared to B. As shown by the superposed signals in E, this region of epidermis contains virtually no K10/K11. In E, the arrow depicts the pronounced hyperkeratosis. Bar, 50 μ m.

Significance of the Phenotype Observed in K16-overexpressing Mice

A priori, the finding that a relatively modest overexpression of a wild-type keratin gene in a tissue-specific fashion led to the formation of multiple small aggregates in transgenic mouse skin keratinocytes is a surprising one. Identifying the mechanism(s) responsible for this accumulation will significantly enhance our understanding of the biology of keratin filaments. Three main possibilities ought to be considered. First, as mentioned above, it could be that human K16 behaved as a (mild) keratin mutant able to perturb mouse endogenous keratin filaments at suitably high intracellular levels. The cloning and characterization of the mouse keratin gene orthologue to human K16 should be informative at that level. Second, it may be that the extent of type I keratin overexpression in transgenic mouse keratinocytes was such that it exceeded the capacity of the posttranscriptional degradation mechanisms thought to maintain a proper balance between type I and type II keratins in the cytoplasm (Kulesh et al., 1989; Lersch et al., 1989), leading to their accumulation as small aggregates. If true, this implies that similar results could have been obtained if the expression of other type I keratins had been placed under the same regulatory sequences. Third, it may be that K16 intrinsically displays unconventional assembly properties, and that its expression to relatively high intracellular levels serves the purpose of promoting a reorganization of the keratin filament network. Preliminary findings stemming from in vitro assembly studies with purified human epidermal keratins provide some insights in that respect. Indeed, we have found that type I-type II heterotypic complexes containing K16 are significantly less stable in urea buffer than those containing K14 as their type I keratin, and moreover, that the efficiency with which K16 assembles into 10-nm filaments with either K5 or K6 as type II partner in vitro is also significantly lower (Paladini, R., and P. A. Coulombe, unpublished observations). The completion of these and additional studies should allow us to better understand the mechanisms responsible for the formation of these small aggregates in the skin keratinocytes of our K16-overexpressing mice.

Given that the type I keratin genes encoding K14, K10, and K9 have been implicated in human genetic skin diseases (Coulombe and Fuchs, 1994), it is possible and even likely that mutations in the K16 gene underlie a human disease as well. We do not know the extent to which the phenotype that could arise from the tissue-specific expression of a dominant negative K16 mutant in transgenic would resemble the one described in the present study, which involved overexpression of the wild-type human K16 gene. Yet, the morphological attributes of lesional skin in our phenotypic transgenic mice showed an intriguing resemblance to those associated with some skin diseases featuring acantholytic dyskeratosis (Waldo and Ackerman, 1978). Among such diseases one finds Darier and Hailey-Hailey diseases, two related genetic skin disorders having an autosomal dominant inheritance (Burge, 1992; Burge and Wilkinson, 1992). Histological examination of the lesions in Hailey-Hailey and especially Darier patients reveals abnormal keratinization and acantholysis in the suprabasal layers. Remarkably, desmosomes are reduced at the keratinocyte cell surface, while those present

are frequently abnormal, with tonofilament detachment and disruption of the plaque at their intercellular domain. It is generally believed that a functional defect in desmosomal component(s) is responsible for both these diseases (Burge, 1992; Burge and Wilkinson, 1992). The Darier disease locus has recently been mapped in several pedigrees to 12q23-24 (Craddock et al., 1993; Bashir et al., 1993; Parfitt et al., 1994), i.e., outside of chromosomal loci known to contain gene clusters encoding known structural components of desmosomes, and several centimorgans away from a large cluster of type II keratin genes located at 12q11-13 (Rosenberg et al., 1991). The Hailey-Hailey locus has yet to be mapped, but linkage to the two major clusters of keratin genes (type I and type II) and to the Darier locus have been ruled out (Welsh et al., 1994). While it appears quite unlikely that Darier and Hailey-Hailey are keratin gene disorders, our data suggest that these diseases may not necessarily involve a structural defect in an integral desmosome component because some of the typifying ultrastructural alterations associated with these diseases were produced by overexpression of a keratin gene in transgenic mouse skin.

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